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A New Orphan Member of the Nuclear Hormone Receptor Superfamily That Interacts with a Subset of Retinoic Acid Response Elements

MYRIAM BAES,† TOD GULICK, HUENG-SIK CHOI, MARIA GRAZIA MARTINOLI,‡ DEVENDRANATH SIMHA, AND DAVID D. MOORE*

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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We have identified and characterized a new orphan member of the nuclear hormone receptor superfamily, called MB67, which is predominantly expressed in liver. MB67 binds and transactivates the retinoic acid response elements that control expression of the retinoic acid receptor β2 and alcohol dehydrogenase 3 genes, both of which consist of a direct repeat hexamers related to the consensus AGGTCA, separated by 5 bp. MB67 binds these elements as a heterodimer with the 9-*cis*-retinoic acid receptor, RXR. However, MB67 does not bind or activate other retinoic acid response elements with alternative hexamer arrangements or any of several other wild-type and synthetic hormone response elements examined. The transactivation of retinoic acid response elements by MB67 is weaker than that conferred by the retinoic acid receptors but does not require the presence of all-*trans* retinoic acid, 9-*cis*-retinoic acid, or any exogenously added ligand. We propose that MB67 plays an important role in the complex network of proteins that govern response to retinoic acid and its metabolites.

The nuclear hormone receptor superfamily is a large group of related transcription factors which includes members that bind a diverse array of ligands, including steroids, thyroid hormone (T3), all-*trans* retinoic acid (RA), 9-*cis*-retinoic acid (9-*cis*-RA), and vitamin D (reviewed in references 4, 8, 19, 23, 35). In mammals, approximately a dozen genes encode these conventional receptors. An even larger number of genes encode proteins known as orphan receptors (recently compiled in references 1 and 32). These orphans are structurally and functionally related to the conventional receptors but do not bind known ligands.

The biological roles of the orphans are generally unknown. Some information is available for a limited number which were initially characterized prior to their identification as superfamily members. HNF-4, for example, was originally identified as a liver-specific basic transcription factor, with binding sites in several genes (59). Similarly, SF-1 was identified as a factor associated with activation of expression of several steroidogenic enzymes (31). However, the majority of the orphans were isolated simply by cross-hybridization with probes derived from conventional receptors, and their functions remain largely undefined. By analogy with the conventional receptors, it has generally been thought that most of the orphans will eventually be demonstrated to be ligand-dependent transcriptional activators. However, recent results demonstrate that several, including HNF-4 (10, 30, 59), SF-1 (43), and NGFI-B/Nur77 (13, 48, 70), are capable of activating transcription in the absence of specifically added hormones or other ligands.

Nearly all of the conventional and orphan members of the nuclear hormone receptor superfamily can be segregated into only two major groups on the basis of a short amino acid sequence called the P box. This motif is both an important determinant of DNA binding specificity and the most strongly conserved subregion of the DNA binding domain (12, 44, 65). The larger P-box group includes the receptors for RA, 9-*cis*-RA, T3, and vitamin D, as well as nearly all of the orphans, while the smaller group includes most of the steroid receptors. As monomers, receptors from the two groups recognize related but distinct hexameric consensus sequences. Since most superfamily members function as dimers, however, wild-type hormone response elements generally include at least two hexamers that match the consensus.

Within the larger group, specificity of response is a complex function of both the precise sequence of such hexamers and variations in their relative spacing and orientation. For example, synthetic DNA sites that consist of direct repeats of consensus hexamers separated by 3, 4, or 5 bp (referred to as DR-3, DR-4, and DR-5) show preferential responses to the vitamin D receptor (VDR), the T3 receptors (TR), and the RA receptors (RAR), respectively (66). Each of these three hormone response elements is bound with high affinity and specificity by a heterodimeric complex consisting of the appropriate receptor and the 9-*cis*-RA receptors (RXR) (9, 24, 28, 36, 71, 73). These heterodimers can also interact with additional elements, however, and response to retinoids is particularly complex. Thus, both RAR-RXR and TR-RXR heterodimers can bind to and activate a synthetic element consisting of inverted repeats of the consensus hexamer with no intervening base pairs (IR-0) (9, 36, 46). The RAR-RXR complex can also activate DR-2 (47, 60) and DR-1 elements (17). DR-1 elements can also be recognized by RXR homodimers (45, 74) as well as a number of other superfamily members (30, 64). Additional arrangements of receptor-binding hexamers are found in wild-type RA response elements (RAREs). The rat growth hormone (rGH) T3RE-RARE complex consists of two directly repeated hexamers followed by an inverted copy (6). All three hexamers contribute to the

* Corresponding author. Phone: (617) 726-5943. Fax: (617) 726-6893.

† Present address: Faculty for Pharmaceutical Sciences, Laboratory for Clinical Chemistry, Campus Gasthuisberg, B-3000 Leuven, Belgium.

‡ Present address: Center for Research in Neuroscience, McGill University, The Montreal General Hospital Research Institute, Montreal, Quebec H3G 1A4, Canada.

response to both RA and T3 (69). An element from the medium-chain acyl coenzyme A dehydrogenase (MCAD) gene also includes three hexamers that match the consensus, arranged as an inverted copy separated by 8 bp from two direct repeats with no spacer (53). RAR-RXR heterodimers bind specifically to the two hexamers separated by 8 bp. This tail-to-tail arrangement is referred to as an everted repeat (ER-8). A similar ER-8 RARE was identified in the γ-F-crystallin gene (63).

Thus, the response of a cell to a particular member of the RAR/TR/orphan subgroup is likely to be a very complex function of the expression of several different superfamily members and the ability of these proteins to interact with a number of distinct types of response elements. In an effort to gain further information on the components of this complex regulatory network, we have isolated several new orphans. Here we report the identification of one such orphan and the characterization of its novel role in the transcriptional response to retinoids.

MATERIALS AND METHODS

Isolation of cDNA clones. A degenerate oligonucleotide directed to the P-box sequence of the TR/RAR/orphan receptor subgroup was used to screen an adult human liver cDNA library (gift of B. Seed) in the plasmid expression vector CDM8. The degeneracy of the probe [5′ TG(C/T)GAGG GITG(C/T)AAGG(G/C)ITT(C/T)TT(C/T)(A/C)G 3′] was reduced by replacing positions with fourfold degeneracy with inosine and inserting G for G/A. Approximately 700,000 colonies were screened by hybridization in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.05% P_i–1% sodium dodecyl sulfate (SDS)–100 mg of salmon sperm DNA per ml with 1.8×10^6 cpm of 5′-^{32}P-end-labeled probe per ml at 42°C. Filters were washed in 6× SSC–0.1% SDS and then in 3.0 M tetramethylammonium chloride–50 mM Tris at 50°C (2). A number of positive clones were isolated and partially characterized by DNA sequencing. MB67 was encoded by two identical clones with 1.5-kb inserts.

Plasmids. A series of thymidine kinase (TK) promoter/chloramphenicol acetyltransferase (CAT) reporter plasmids with various response elements was constructed by insertion of oligonucleotides into the vector pUTKAT3 (52). Most were as previously described by Williams et al. (69), except for those containing response elements from osteocalcin (14) and MCAD (52). In the TK promoter-luciferase reporters, the CAT reporter gene is replaced by the luciferase gene. Different numbers of copies of the βRARE oligonucleotide or a single copy of a synthetic DR-4 T3RE were inserted upstream of the TK promoter. The original full-length isolate of the MB67 cDNA in the CDM8 vector (57) was used for expression of the intact MB67 protein. The RARβ expression vector included the coding region inserted into CDM8 (57) with a consensus translation initiation site. The TRβ1 expression vector was as previously described (7). The TR/MB67, glucocorticoid receptor (GR)/MB67, and TR/GR chimeras were constructed by using PCR with primers flanking the A, B, and C domains of TR or GR and the D, E, and F domains of MB67 or GR. At the N terminus of the D domain of the TR/MB67 chimera, a Lys residue of MB67 was replaced by Ala. Bacterial expression vectors were modified versions of a bacteriophage T7 RNA polymerase expression vector (2). For Myc/MB67, the c-Myc 9E10 epitope (EQKLISEEDLN [18]) was inserted between a translation initiator consensus sequence and the MB67 coding region. The influenza virus epitope, YPYDVPDYA, was also used analogously.

Cell culture and transfections. JEG3 and other cells were plated in 6-cm-diameter dishes and grown for 24 h in Dulbecco modified Eagle medium supplemented with 10% charcoal-stripped fetal bovine serum prior to transfection. Transfections were carried out by using either calcium phosphate or DEAE-dextran with various amounts of CAT or luciferase reporter plasmid, receptor expression vector, and the internal control plasmid pTKGH (58). Cellular CAT activities were assayed after 2 days and normalized to medium human growth hormone (hGH) concentrations as described previously (7). Luciferase activity was assayed by using reagents and procedures from Promega (Madison, Wis.). Transfections using luciferase reporters were also normalized to hGH expression from the pTKGH internal control.

Protein production and gel shift analysis. Both Myc/MB67 and native RARα were overexpressed in *Escherichia coli* BL21(DE3). Cells containing the expression vectors were induced at 30°C with 0.4 mM isopropylthiogalactopyranoside (IPTG), and expressed proteins were partially purified by ammonium sulfate precipitation as described previously (49). A vaccinia virus vector directing expression of RXRα was constructed using standard methods (2), and nuclear extracts were prepared from infected HeLa cells as described previously (2). For gel shift analysis, double-stranded oligonucleotides were labeled by filling in with [^{32}P]dCTP and Klenow DNA polymerase or [^{32}P]γATP and polynucleotide kinase. Proteins and antibody were preincubated at 25°C for 30 min and were then added to a reaction mixture consisting of 10 mM Tris (pH 8.0), 1 mM dithiothreitol, 12% glycerol, 80 mM KCl, 0.5 mM EDTA, 0.5 μg of poly(dI-dC), and 25,000 cpm of probe in a total volume of 20 μl. Bound and unbound probe were resolved on 4% polyacrylamide gels in 0.5× Tris-borate as described previously (2).

RESULTS

Isolation of MB67 cDNA. A human liver cDNA library was screened with a degenerate oligonucleotide based on the sequence of the P-box region of the DNA binding domain of the RAR/TR/orphan class. Among a number of hybridizing clones isolated, two that contained sequences distinct from those of known members of the nuclear hormone receptor superfamily were chosen for further analysis. Determination of the complete DNA sequence of each revealed that both encoded a novel superfamily member, referred to as MB67. The sequence flanking the first AUG codon of the open reading frame indicated in Fig. 1 is a good match to the consensus for translation initiators (29). This putative initiator codon is preceded by a relatively long 5′ untranslated region that includes an in-frame terminator 42 nucleotides upstream. As observed for a number of other members of the superfamily, several additional upstream AUGs initiate short open reading frames.

MB67 is one of the smallest members of the superfamily (348 amino acids), with a particularly short N-terminal domain, but it contains all of the most highly conserved sequence motifs shared by superfamily members. Thus, the DNA binding domain includes a P-box sequence identical to that of the RARs, TRs, and RXRs. The putative ligand binding and dimerization domain of MB67 includes sequences that match three conserved subregions found in both orphan and conventional members of the superfamily (56), but the orphan shows only a limited match to a C-terminal sequence associated with ligand-dependent transcriptional activation in some receptors

A

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GTGAGCTTGCTCCTTAAGTTACAGGAACTCTCCTTATAATAGACACTTCATTTTCCTAGTCCATCCCTCATGAAA

AATGACTGACCACTGCTGGGCAGCAGGAGGGATGATAATCCTAACTCCAATCACTGGCAACTCCTGAGATCAGAG

GAAAACCAGCAACAGCGTGGGAGTTTGGGGAGAGGCATTCCATACCAGATTCTGTGGCCTGCAGGTGACATGCTG

                                                    M  A  S  R  E  D  E  L  R  N
CCTAAGAGAAGCAGGAGTCTGTGACAGCCACCCCAACACGTGACGTCATGGCCAGTAGGGAAGATGAGCTGAGGA

  C  V  V  C  G  D  Q  A  T  G  Y  H  F  N  A  L  T  C  E  G  C  K  G  F  F
ACTGTGTGGTATGTGGGGACCAAGCCACAGGCTACCACTTTAATGCGCTGACTTGTGAGGGCTGCAAGGGTTTCT

  R  R  T  V  S  K  S  I  G  P  T  C  P  F  A  G  S  C  E  V  S  K  T  Q  R
TCAGGAGAACAGTCAGCAAAAGCATTGGTCCCACCTGCCCCTTTGCTGGAAGCTGTGAAGTCAGCAAGACTCAGA

  R  H  C  P  A  C  R  L  Q  K  C  L  D  A  G  M  R  K  D  M  I  L  S  A  E
GGCGCCACTGCCCAGCCTGCAGGTTGCAGAAGTGCTTAGATGCTGGCATGAGGAAAGACATGATACTGTCGGCAG

  A  L  A  L  R  R  A  K  Q  A  Q  R  R  A  Q  Q  T  P  V  Q  L  S  K  E  Q
AAGCCCTGGCATTGCGGCGAGCAAAGCAGGCCCAGCGGCGGGCACAGCAAACACCTGTGCAACTGAGTAAGGAGC

  E  E  L  I  R  T  L  L  G  A  H  T  R  H  M  G  T  M  F  E  Q  F  V  Q  F
AAGAAGAGCTGATCCGGACACTCCTGGGGGCCCACACCCGCCACATGGGCACCATGTTTGAACAGTTTGTGCAGT

  R  P  P  A  H  L  F  I  H  H  Q  P  L  P  T  L  A  P  V  L  P  L  V  T  H
TTAGGCCTCCAGCTCATCTGTTCATCCATCACCAGCCCTTGCCCACCCTGGCCCCTGTGCTGCCTCTGGTCACAC

  F  A  D  I  N  T  F  M  V  L  Q  V  I  K  F  T  K  D  L  P  V  F  R  S  L
ACTTCGCAGACATCAACACTTTCATGGTACTGCAAGTCATCAAGTTTACTAAGGACCTGCCCGTCTTCCGTTCCC

  P  I  E  D  Q  I  S  L  L  K  G  A  A  V  E  I  C  H  I  V  L  N  T  T  F
TGCCCATTGAAGACCAGATCTCCCTTCTCAAGGGAGCAGCTGTGGAAATCTGTCACATCGTACTCAATACCACTT

  C  L  Q  T  Q  N  F  L  C  G  P  L  R  Y  T  I  E  D  G  A  R  V  G  F  Q
TCTGTCTCCAAACACAAAACTTCCTCTGCGGGCCTCTTCGCTACACAATTGAAGATGGAGCCCGTGTGGGGTTCC

  V  E  F  L  E  L  L  F  H  F  H  G  T  L  R  K  L  Q  L  Q  E  P  E  Y  V
AGGTAGAGTTTTTGGAGTTGCTCTTCACTTCCATGGAACACTACGAAAACTGCAGCTCCAAGAGCCTGAGTATG

  L  L  A  A  M  A  L  F  S  P  D  R  P  G  V  T  Q  R  D  E  I  D  Q  L  Q
TGCTCTTGGCTGCCATGGCCCTCTTCTCTCCTGACCGACCTGGAGTTACCCAGAGAGATGAGATTGATCAGCTGC

  E  E  M  A  L  T  L  Q  S  Y  I  K  G  Q  Q  R  R  P  R  D  R  F  L  Y  A
AAGAGGAGATGGCACTGACTCTGCAAAGCTACATCAAGGGCCAGCAGCGAAGGCCCCGGGATCGGTTTCTGTATG

  K  L  L  G  L  L  A  E  L  R  S  I  N  E  A  Y  G  Y  Q  I  Q  H  I  Q  G
CGAAGTTGCTAGGCCTGCTGGCTGAGCTCCGGAGCATTAATGAGGCCTACGGGTACCAAATCCAGCACATCCAGG

  L  S  A  M  M  P  L  L  Q  E  I  C  S  *
GCCTGTCTGCCATGATGCCGCTGCTCCAGGAGATCTGCAGCTGAGGCCATGCTCACTTCCTTCCCCAGCTCACCT

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TTGCAATGAAAGACTAAAGCAAAAC
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B

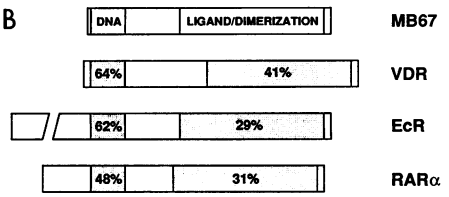

FIG. 1. A. Nucleotide and predicted amino acid sequences of MB67. The amino acid sequence of the DNA binding domain is in bold type. The sequence of the DNA binding specificity-determining P box (underlined) is identical to that of the TRs, RARs, and RXRs. Conserved subregions within the ligand binding domain (56) are also underlined. (B) Relationship of MB67 to other superfamily members. The percent amino acid sequence identity in comparisons of the DNA and ligand/dimerization domains of MB67 with those of the human VDR, the *Drosophila melanogaster* ecdysone receptor (EcR), and the human RARα is indicated. See reference 1 and 32 for extensive comparisons of the sequences of superfamily members.

(11). MB67 is the third member of a divergent subgroup within the superfamily that also includes the vitamin D receptor (VDR) and ecdysone receptor (Fig. 1B) (1, 32). MB67 is approximately as closely related to the VDR as the RARs are to the TRs.

MB67 mRNA is expressed preferentially in liver, with much lower levels in other tissues. As shown in Fig. 2, liver expresses a broad band of approximately 1.4 to 1.7 kb which is not well resolved from an additional species of approximately 2.1 kb, as well as a third species of approximately 2.9 kb. On longer exposures, much lower levels of an approximately 7-kb transcript are seen in liver, and low levels of an approximately

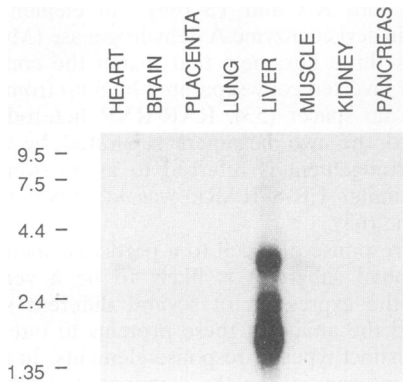

FIG. 2. Distribution of MB67 mRNA in human tissues. A Northern (RNA) blot containing 2 μg of poly(A)$^+$ RNA from the indicated human tissues (Clontech) was hybridized at high stringency with an MB67 probe containing the hinge and putative ligand binding (D and E) domains. Positions and sizes (in kilobase) of molecular weight markers are indicated. In much longer exposures of the same blot, low levels of specific hybridization were observed for an approximately 3-kb transcript in heart, muscle, brain, kidney, and lung. Kidney also expressed an approximately 1.6-kb transcript.

3.0-kb transcript are also observed in heart and muscle, with even lesser amounts in kidney and lung. Kidney also expresses low levels of an approximately 1.6-kb species. A generally similar distribution is observed in murine tissues. The different MB67 mRNAs observed in both human and murine tissues could arise from a variety of mechanisms. However, a more extensive analysis of the murine mRNAs, to be presented elsewhere, demonstrates that identical patterns are generated by 5′ untranslated and coding-region probes, suggesting that this variability is not a consequence of divergent 5′ untranslated and N-terminal regions as observed, for example, with TR (26) and RAR (37, 38, 72) isoforms.

Transactivation by MB67. As an initial step in the characterization of the function of MB67, its potential ligand binding domain was fused to the DNA binding domain of TRβ1 to generate a TR/MB67 chimera similar to those used to characterize several other nuclear receptors (20, 22, 27). Vectors expressing this chimera or intact TRβ1 were cotransfected with a T3RE/CAT reporter plasmid into JEG3 choriocarcinoma cells. Transfections were carried out in medium supplemented with fetal bovine serum treated with activated charcoal to remove T3 and other small hydrophobic molecules that might act as ligands. As expected, the native TR directed strong T3-dependent transcriptional activation (Fig. 3). In contrast, the TR/MB67 chimera activated expression of T3RE/CAT in the absence of any exogenously added ligand. The level of TR/MB67-mediated transactivation was 60% of that produced by TRβ in the presence of a saturating amount of T3. Analogous results were obtained with a similarly constructed GR/MB67 chimera in cotransfections with a glucocorticoid-responsive mouse mammary tumor virus/CAT reporter. In these experiments, a TR/GR chimera showed glucocorticoid-dependent induction of the T3RE/CAT reporter similar to that described for an analogous chimera (62), confirming the appropriate response of this series of chimeras and the validity of this approach.

To exclude the possibility that the apparently constitutive activity of the MB67 ligand binding domain was a consequence of an activator present even in charcoal-stripped serum, similar cotransfections were carried out in serum-free medium. Under

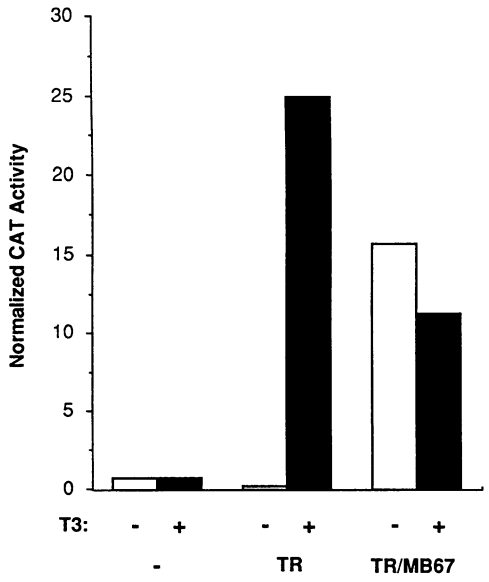

FIG. 3. Constitutive activity of a TR/MB67 chimera. TK35BA, a derivative of pUTKAT3 containing two copies of an up-mutant version of the rGH TRE (6), was cotransfected with control vector or a vector expressing either intact TRβ1 or TR/MB67, a chimera including the N-terminal and DNA binding domains of TR and the hinge and the putative ligand and dimerization domains of MB67. Transfections of JEG3 cells were carried out in Dulbecco modified Eagle medium containing 10% charcoal-stripped fetal bovine serum and either 10 nM T3 or no supplement, as indicated.

these conditions, overall levels of reporter gene expression were reduced, but the TR/MB67-mediated transactivation remained substantial (23% of the T3-activated TR level).

Given the activation observed with the MB67 chimeras, a vector expressing native MB67 was cotransfected with a number of CAT reporter plasmids containing a wild-type or synthetic RARE, T3RE, VDRE, or estrogen response element (ERE) (Table 1). As with the chimeras, transfections were carried out in the presence of medium containing charcoal-stripped serum. Significant transactivation by MB67 was observed only with the reporters containing RAREs of the DR-5 type from the promoters controlling expression of the RARβ2 isoform (referred to as the βRARE) (15, 61, 68) and alcohol dehydrogenase 3 (ADH3) (16). Other RAREs that are not of the DR-5 type were unresponsive. These constructs included one with two copies of an up-mutant version of the rGH RARE/T3RE (RARE/T3RE/rGHup) that was induced more than 30-fold by ligand-activated RARs or TRs in parallel cotransfections (not shown), as well as the DR-2 elements from the ApoA1 and CRBPI genes and the ER-8 element from the MCAD gene. In some experiments, a modest response was observed with the artificial palindromic T3RE/RARE (RARE/T3RE/pal) and the VDRE/RARE from the osteocalcin gene (VDRE/rOST).

The level of transactivation of the single-copy βRARE and ADH3 elements by MB67 is only 5 to 10% of the potent response conferred by the RARs. However, the response of these single-copy elements to MB67 is similar to that observed with many other hormone response elements and their cognate receptors. For example, the three- to sixfold activation by MB67 is comparable to that conferred by the TRs or RARs in analogous cotransfections with the wild-type rGH or laminin T3RE/RAREs and is greater than that observed with the VDR and single copies of the osteocalcin VDRE (2a, 5, 14, 67, 69). To confirm the ability of MB67 to activate the βRARE, it was cotransfected with luciferase reporters containing multiple copies of that element. In the experiment shown in Fig. 4, MB67 transactivated a reporter containing three copies of that element by approximately 30-fold. By comparison, RARβ had no effect on expression in the absence of ligand but conferred more than 200-fold activation in the presence of RA.

The activation of the βRARE by MB67 was observed in several cell types and in a variety of culture conditions. In addition to JEG3, the cell lines tested included CV1 monkey kidney fibroblasts, HepG2 human hepatoma cells, and the human lung tumor-derived cell line H661. MB67 activation was unaffected by addition of a variety of compounds considered potential ligands, including 1,25-dihydroxyvitamin D and several vitamin D analogs, several steroids and hydroxycholesterols, ciprofibrate, and vitamin E. Although MB67 shows

TABLE 1. MB67 activates a subset of RAREs[a]

Element	Sequence	Fold induction
RARE/hRARβ	ggtag**GGTTCA**ccgaa**AGTTCA**ctct	6.4
RARE/hADH3	ctgaaTGACCCaaagggaaaac**TGAACT**ctgaa**TGACCC**ctg	3.0
RARE/hApoA1	ac**TGAACC**ct**TGACCC**c**TGCCCT**g	0.7
RARE/CRBPI	agt**AGGTCA**aaa**AGGTCA**gaca	1.1
RARE/MCAD	gggtt**TGACCT**ttctctcc**GGGTAAAGGTGA**aggc	1.2
RARE/T3RE/pal	tc**AGGTCATGACCT**ga	1.5
RARE/T3RE/rGHup	**AGGTAA**gatc**AGGGAC**g**TGACCT**	1.0
RARE/T3RE/laminin	agacaggt**TGACCC**ttttttctaagggct**TAACCT**agc**TCACCT**c	0.8
T3RE/rME	aggacgttg**GGGTTA**gggg**AGGACA**gtg	1.0
T3RE/rMHC	ctgg**AGGTGA**cagg**AGGACA**gcagccctga	1.0
VDRE/rOST	t**GGGTGA**atg**AGGACA**ttacTGACCGctccg	1.4
ERE/pal	tc**AGGTCA**ctg**TGACCT**ga	1.1

[a] A series of previously described reporter plasmids (6, 14, 52, 69) in which the indicated response elements are inserted upstream of the TK promoter were cotransfected with an MB67 expression vector. All reporters contain single copies of the response elements, except for rGHup (two copies) and rOST (four copies). All sequences are listed 5′ to 3′ as they occur in the context of their native promoters. For each element, sense or antisense hexamers with exact or partial matches to the consensus AGGTCA hexamer recognized by the TR/RAR/orphan P-box class are indicated by capital letters; those hexamers for which there is clear evidence of a functional role in a specific response are indicated in bold type. Three micrograms of an MB67 expression vector or the parent vector CDM8 was cotransfected with 6 μg of the indicated reporters or pUTKAT3 and 5 μg of the pTKGH internal control into JEG3 cells under conditions as described in Materials and Methods. Fold induction corresponds to hGH-normalized CAT activity with CDM8-MB67 transfection compared with that of the CDM8 vector alone or to activation of the specific response element by MB67 compared with TK. Results are means of at least two independent transfections.

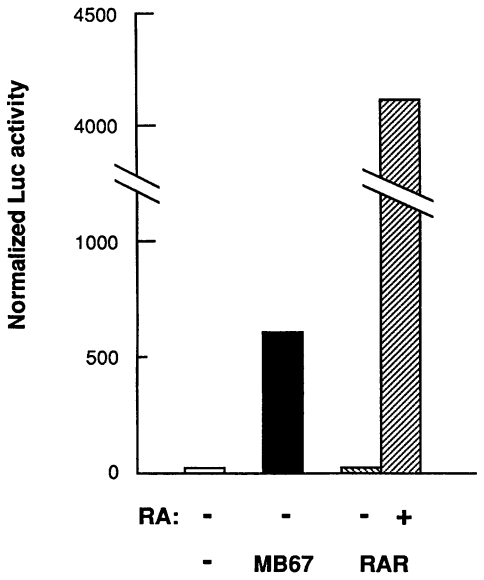

FIG. 4. Activation of a reporter containing multiple copies of the βRARE by MB67 and RAR. A plasmid with three copies of the βRARE inserted upstream of a TK promoter/luciferase reporter construct was cotransfected with either a control expression vector (−) or an MB67 (1 μg) or RARβ (0.1 μg) expression vector. The RARβ transfection was carried out in the presence or absence of 10^{-6} M RA, as indicated. Transfections of HepG2 cells were carried out in the presence of charcoal-stripped serum. Relative luciferase (Luc) activity was determined by normalization to levels of hGH expression directed by the pTKGH internal control.

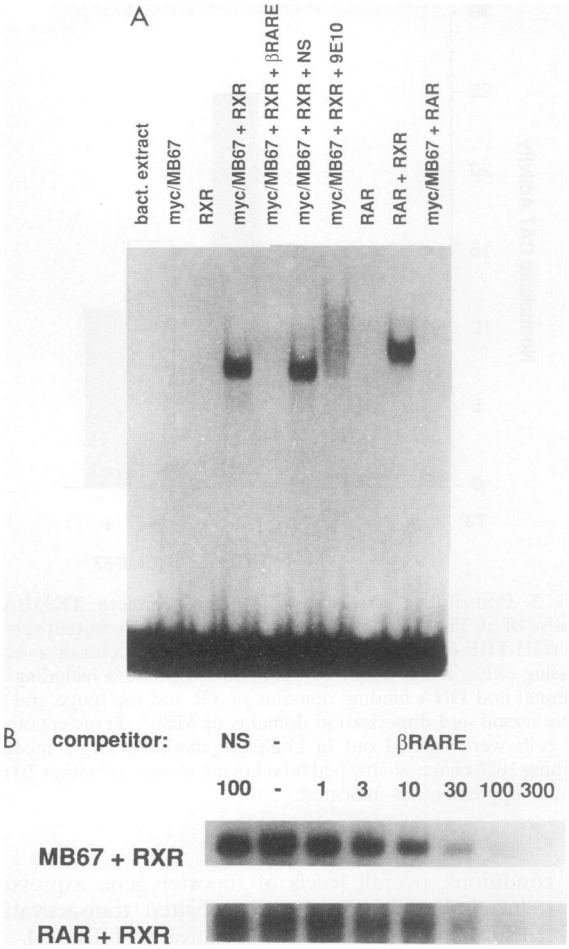

FIG. 5. MB67 DNA binding. (A) Heterodimers of MB67 and RXRα bind to the βRARE. As indicated, epitope-tagged MB67 (Myc/MB67), RARα, and RXRα were used in electrophoretic mobility shift assays with a βRARE probe in the presence or absence of a 100-fold excess of specific (βRARE) or nonspecific (NS) oligonucleotide competitor DNA, or with the 9E10 monoclonal antibody recognizing the c-Myc epitope (18). Both Myc/MB67 and native RARα were overexpressed in *E. coli*, and RXRα was expressed in vaccinia virus (2). Identical results were obtained with bacterially prepared RXR. (B) Relative binding affinity of MB67-RXR and RAR-RXR heterodimers. Binding reaction mixtures containing a constant amount of bacterially produced RXRα and equivalent amounts of either Myc/RARα or Myc/MB67 were incubated with either no competitor, nonspecific competitor, or increasing amounts of an unlabeled βRARE competitor oligonucleotide, as indicated. Only the retarded complexes are shown.

complex interactions with the retinoid receptors as described below, neither RA nor 9-*cis*-RA showed specific effects on the activity of MB67 on the βRARE.

Because the estrogen and progesterone receptors can be activated by second messenger pathways in the absence of their steroid ligands (51) and the activity of the orphan COUP-TF may be modulated similarly (50), a number of approaches were taken to determine whether MB67 is responsive to known signalling pathways. In the presence of charcoal-stripped serum, MB67 function was not affected by stimulation of protein kinase A activity by forskolin or 8-bromo-cyclic AMP (cAMP) or by inhibition of protein kinase A by cotransfection with a vector expressing the protein kinase inhibitor peptide. Stimulation or down regulation of protein kinase C activity by short- or long-term treatments with phorbol esters was without effect, as was a combined stimulation with 8-bromo-cAMP plus a short term treatment with tetradecanoyl phorbol acetate. Finally, inhibition of phosphatase activity by treatment with okadaic acid was also ineffective.

DNA binding by MB67. To characterize binding of MB67 to the wild-type hormone response elements that it transactivates, interactions of MB67 with the βRARE were examined in detail. On the basis of the binding of that element by RAR-RXR heterodimers, epitope-tagged and untagged derivatives of RXRα, RARα, and MB67 were overexpressed in both *E. coli* and vaccinia virus-based systems. As demonstrated in Fig. 5A, no high-affinity binding to the βRARE was observed with the epitope-tagged Myc/MB67 or with RXR or RAR alone. The combination of Myc/MB67 with RAR also showed no binding. However, a prominent retarded complex was observed when Myc/MB67 and RXR were combined. This complex was specifically competed for by an unlabeled βRARE oligonucleotide, and its mobility was further retarded by the 9E10 monoclonal antibody specific for the epitope tag (18), confirming the presence of MB67 in the complex. No such supershift was observed with a nonspecific antibody (not shown). As expected, the combination of the RAR and RXR proteins also generated a strong complex that migrated somewhat more slowly than the MB67-RXR complex. Analogous results were observed with the ADH3 RARE. However, when different synthetic DR-5 elements containing two consensus AGGTCA hexamers were tested, significant variations in binding of MB67-RXR heterodimers were observed. The contribution of base pairs outside the hexameric consensus to MB67 binding is under investigation.

In agreement with the results of cotransfections, MB67 did not bind RAREs not of the DR-5 class, in either the presence or absence of RXR. The elements tested included the wild-type DR-2 RARE from the ApoAI gene, the wild-type ER-8 element from the MCAD gene, and synthetic DR-1 and DR-2 elements.

The relative amounts of the MB67-RXR and RAR-RXR shifted complexes in Fig. 5A are similar, suggesting that the two heterodimeric complexes have similar affinities for the βRARE. To test this directly, binding reactions were carried out in which a constant amount of RXR was combined with equivalent amounts of either Myc/MB67 or Myc/RAR, as assessed by Western blotting (immunoblotting) of bacterial extracts with the 9E10 antibody. As indicated in Fig. 5B, addition of increasing amounts of the unlabeled βRARE to either combination generated very comparable competition curves. Parallel results were also obtained with MB67 and RAR proteins containing the influenza virus epitope tag. We conclude that the affinities of the MB67-RXR and RAR-RXR complexes for this element are similar.

Functional interactions of MB67 with retinoid receptors. Support for the functional significance of the RXR-MB67 interaction was obtained in cotransfections using both the intact RXRα and a derivative deleted for the N-terminal and DNA binding domains (dnRXR). This mutant receptor is predicted to act as a dominant inhibitor of the function of RXR heterodimer partners as a consequence of formation of heterodimers that contain only a single DNA binding domain and are unable to recognize specific response elements with high affinity. As shown in Fig. 6A, the combination of MB67 and the intact RXR transactivated the three-copy βRARE reporter more than 100-fold. The experiment in Fig. 6B demonstrates that MB67-mediated activation of the βRARE-containing reporter was strongly inhibited by coexpression of the dnRXR, as was a similar activation of a T3-responsive reporter by TRβ.

Although MB67 and RAR did not show direct interaction in vitro, potential functional interactions between them were examined in cotransfections of the single-copy βRARE reporter with various doses of both MB67 and RAR expression vectors. In cotransfections containing a constant, subsaturating dose of RARβ vector, increasing doses of the MB67 vector resulted in a substantial decline in the overall RA induction ratio (Fig. 7A). This was a consequence of both the increase in basal (no RA) expression directed by MB67 and a moderate decrease in the level of RA-induced expression (Fig. 7B). In reciprocal experiments in which increasing amounts of RARβ expression vector were added to a constant amount of MB67 vector in the absence of RA, a blunting of the activation effects of MB67 was observed. The inhibitory effect of higher doses of MB67 on RA response was even more marked with the three-copy βRARE reporter; the substantial increase in basal expression directed by MB67 decreased the fold RA induction to less than 5% of that observed in its absence. Overall, these results are consistent with the DNA binding results and suggest competitive occupation of the element by MB67-RXR and RAR-RXR complexes in vivo.

DISCUSSION

We have isolated a novel orphan member of the nuclear receptor superfamily and characterized both its DNA binding properties and its functional effects on a number of hormone response elements. The biochemical results demonstrate that MB67 binds as a heterodimer with RXR to the βRARE and the ADH3 RARE, both direct repeats of the hexamers related

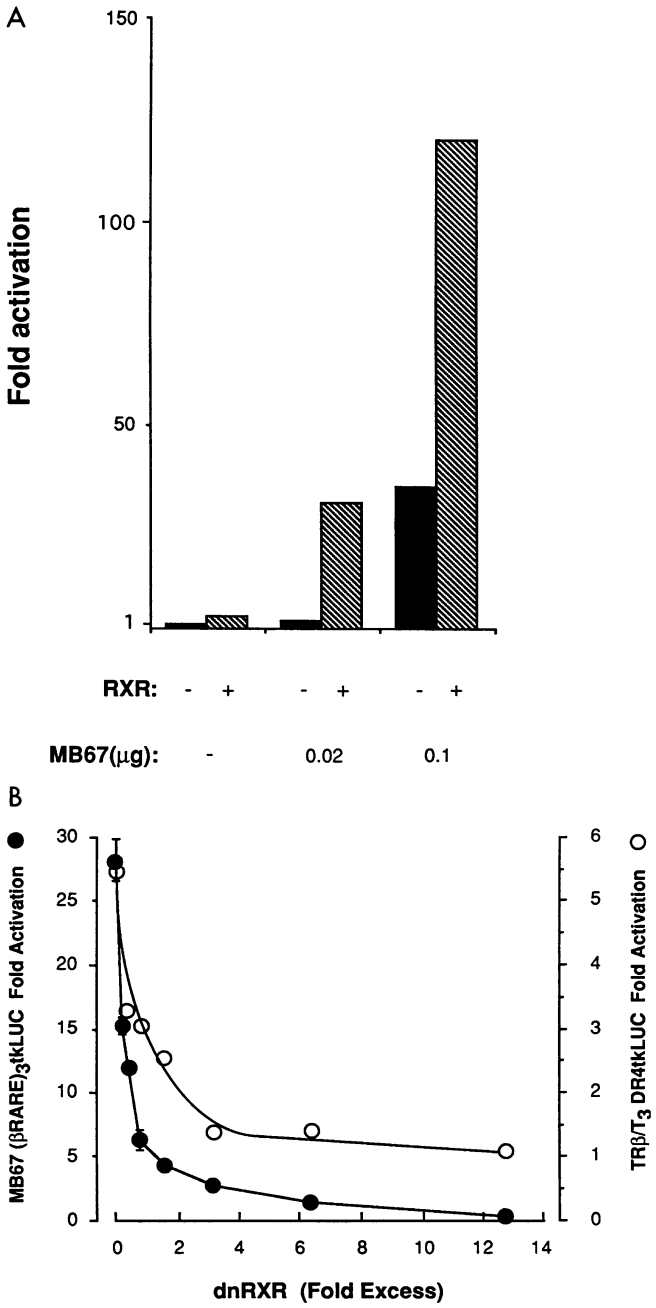

FIG. 6. Functional interaction of MB67 with RXR. (A) Stimulation of MB67 transactivation by RXR. The indicated amounts of a vector expressing intact MB67 or 0.1 μg of an RXR vector was cotransfected into HepG2 cells with the $\beta RARE_3$ TK/Luc reporter and the pTKGH internal control. (B) Inhibition of MB67 and TR transactivation by a dominant negative RXR. HepG2 cells were cotransfected with the increasing amounts of vector expressing dnRXR, a dominant negative derivative of RXRα starting from a methionine two residues from the C terminus of the DNA binding domain. Cotransfections included an MB67 (1.0 μg) or TRβ (0.1 μg) expression vector with the indicated molar ratios of dnRXR vector, the three-copy βRARE/TK/LUC reporter or a DR-4/TK/LUC reporter, and the pTKGH internal control; a constant amount of transfected expression vector DNA was maintained by addition of the CDM8 parent vector. MB67 fold activation represents the ratio of expression in the presence of MB67 vector to that in the presence of CDM8 alone. TR fold activation represents the ratio of expression in the presence of T3 (10 nM) to expression in its absence.

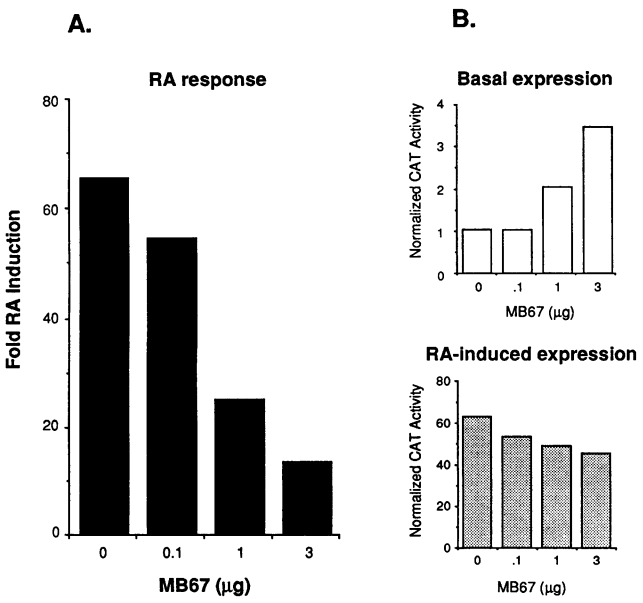

FIG. 7. Combined effects of MB67 and RARβ on the βRARE. (A) MB67 decreases the RA induction ratio. For various doses of MB67 expression vector, the effect of MB67 on relative induction of CAT expression by RA is shown. (B) Effect of MB67 on basal and RA-induced expression. For the same results, the effects of various doses of MB67 on levels of basal or RA-induced expression are shown. JEG3 cells were cotransfected with various amounts of MB67 expression vector and a subsaturating dose of RARβ vector (20 ng) together with the single-copy βRARE/TK/CAT construct and pTKGH and incubated in the presence or absence of 10^{-6} RA. The total amount of expression vectors was kept constant by adding CDM8 vector as necessary to make the total of CDM8 plus MB67 vector equal to 3 μg.

to the AGGTCA consensus separated by 5 bp (DR-5). In cotransfections, MB67 transactivates these two RAREs in the absence of retinoids or any other exogenously added ligands. Both the stimulatory effects of intact RXR and the inhibitory effects of a dominant negative RXR derivative indicate that MB67-RXR heterodimers are responsible for this transactivation. Some previous reports (34, 68) have described activation of RAREs by cotransfected RARs in the absence of added retinoids. In agreement with most other reports, however, we have not consistently observed such effects with RARα or RARβ, even with sensitive multicopy reporters (Fig. 4). In contrast to its effects on the DR-5 elements, MB67 does not bind or transactivate other classes of RAREs or any other elements tested. We conclude that MB67 is a new heterodimer partner for RXR that acts as a specific, retinoid-independent activator of a subset of RAREs.

Although the affinities of MB67-RXR and RAR-RXR heterodimers for the βRARE are closely comparable in vitro, the 3- to 6-fold activation of a single copy of that element by MB67 is modest by comparison with the unusually strong activation (50- to 100-fold) observed with RARs. This could be a consequence of any of a number of mechanisms, including relatively inefficient expression of the MB67 protein or an inherently lower transactivation function for the orphan. The former possibility is supported by the fact that doses of MB67 expression vectors required for half-maximal transactivation are higher than those observed with expression vectors for other receptors. The latter possibility is supported by the facts that (i) the levels of activation directed by saturating doses of MB67 vector are still relatively low, at least by comparison with those for RARs, and (ii) MB67 essentially lacks the N-terminal A/B domain, which contains sequences necessary for full transcriptional activation function in RARs and a number of other superfamily members.

The decreased relative activity of MB67 could also simply reflect the lack of sufficient levels of an appropriate ligand. However, several considerations suggest that the effects of MB67 described here may represent truly ligand-independent functions. Transactivation by MB67 is observed in the absence of serum or any exogenously added ligand, is not dependent on a particular cell type, and is not specifically affected by supplementation with various candidate ligands or by treatments that alter various second messenger pathways. Moreover, we have recently used a yeast genetic system to identify a protein that interacts specifically with the ligand binding domain of either TR or RXR, but only in cells grown in the presence of their cognate ligands (33). In yeast cells growing in minimal medium, no such additions are necessary for interaction of this protein with MB67. An increasing number of reports demonstrate that several other orphans can regulate transcription in the absence of any specifically added ligand (10, 13, 30, 42, 43, 48, 50, 59, 70). Both the inhibition of expression associated with TRs in the absence of ligand (5, 21, 55) and the activation of the progesterone and estrogen receptors by second messenger pathways in the absence of added steroids (51) provide clear examples of ligand-independent effects of conventional receptors. We believe that the assumption of ligand dependence places unnecessary limits on the potential functions of members of the superfamily.

Regardless of whether it has additional, ligand-dependent effects, the retinoid-independent activities of MB67 suggest an alternative explanation for the activation of the βRARE in transgenic reporter constructs designed to identify regions in mouse embryos containing activated RARs (3, 54). The activities of a murine MB67 homolog could also be relevant to the surprisingly modest developmental effects of inactivating RAR genes in mice (39–41). The retinoid independence of MB67 effects also suggests the specific hypothesis that the orphan acts to stimulate expression of a subset of retinoid-responsive genes when RA levels are low. In liver, for example, MB67 activation of the βRARE could contribute to the basal level of expression of RARβ2 mRNA observed in retinol-deficient animals (25). As indicated by the cotransfection results, MB67 could also exert more complex effects on retinoid or other responses. Thus, high levels of MB67 could compete directly with activated RARs for occupancy of DR-5 elements. Sequestration of RXRs by MB67 could also have indirect effects on the activities of RARs or other heterodimer partners. Further analysis will be necessary to define the role of MB67 in the complex network of responses to the nuclear hormone receptor superfamily.

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